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Propensity for HBZ-SP1 isoform of HTLV-I to inhibit c-Jun activity correlates with sequestration of c-Jun into nuclear bodies rather than inhibition of its DNA-binding activity

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ABSTRACT

HTLV-I bZIP factor (HBZ) contains a C-terminal zipper domain involved in its interaction with c-Jun. This interaction leads to a reduction of c-Jun DNA-binding activity and prevents the protein from activating transcription of AP-1-dependent promoters. However, it remained unclear whether the negative effect of HBZ-SP1 was due to its weak DNA-binding activity or to its capacity to target cellular factors to transcriptionally-inactive nuclear bodies. To answer this question, we produced a mutant in which specific residues present in the modulatory and DNA-binding domain of HBZ-SP1 were substituted for the corresponding c-Fos amino acids to improve the DNA-binding activity of the c-Jun/HBZ-SP1 heterodimer. The stability of the mutant, its interaction with c-Jun, DNA-binding activity of the resulting heterodimer, and its effect on the c-Jun activity were tested. In conclusion, we demonstrate that the repression of c-Jun activity *in vivo* is mainly due to the HBZ-SP1-mediated sequestration of c-Jun to the HBZ-NBs.

Introduction

Human T-cell leukemia virus type I (HTLV-I) is an oncoretrovirus involved in the development of an often fatal form of leukemia termed adult T-cell leukemia (ATL) (Uchiyama et al., 1977). Moreover, HTLV-I is the etiologic agent of a neurological demyelinating disease known as tropical spastic paraparesis/HTLV-I-associated myelopathy (Gessain et al., 1985; Osame et al., 1986). Among the proteins encoded by the viral genome, the Tax protein plays a key role in HTLV-I-induced ATL by stimulating the proliferation and the transformation of HTLV-I-infected T cells (Barbeau and Mesnard, 2007; Matsuoka and Jeang, 2007). Tax is also involved in the stimulation of viral protein production by *trans*-activating proviral transcription. Tax interacts with different members of the activating transcription factor/cAMP response element-binding (ATF/CREB) protein family of basic leucine zipper (bZIP) transcription factors and recruits transcriptional cofactors such as p300 and CREB-binding protein (CBP), forming a nucleoprotein complex on the viral promoter located in the 5′ long terminal repeat (LTR) (Gachon et al., 2001, 2002; Kashanchi and Brady, 2005; Lemasson et al., 2002).

The 3′ LTR also contains a functional promoter (Landry et al., 2009; Yoshida et al., 2008) controlling antisense transcription (Cavanagh et al., 2006; Larocca et al., 1989; Murata et al., 2006). Various spliced and unspliced transcripts initiate from the 3′ LTR (Yoshida et al., 2008) allowing the synthesis of two protein isoforms of HBZ (Cavanagh et al., 2006; Murata et al., 2006). However, the most abundant transcripts detected in ATL cell lines and in cells isolated from infected patients correspond to the spliced variant (Cavanagh et al., 2006; Usui et al., 2008). The HBZ isoforms share about 95% amino acid sequence identity and differ only at their N-terminal end (Cavanagh et al., 2006; Murata et al., 2006): 7 amino acids specific for the unspliced HBZ isoform and 4 amino acids specific for the spliced transcript derived HBZ isoform termed here HBZ-SP1. Both isoforms act as repressors of HTLV-I transcription although Tax-mediated transcriptional activation

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through the 5′ LTR is suppressed much more strongly by HBZ-SP1 than that by HBZ, this difference being due to a higher stability of the HBZ-SP1 isoform (Yoshida et al., 2008). HBZ-SP1 down-regulates viral transcription by interacting with nuclear Tax partners such as ATF/CREB proteins, p300, and CBP (Clerc et al., 2008; Lemasson et al., 2007). Consequently, Tax can no longer recruit p300/CBP on the viral promoter and is then unable to *trans*-activate HTLV-I transcription. By using HTLV-I viruses producing defective HBZ, it has been shown that *HBZ* is necessary to enhance HTLV-I infectivity and persistence in infected rabbits (Arnold et al., 2006), an observation which might be consequential to the down-regulating ability of the HBZ isoforms on Tax-dependent transcription (Mesnard et al., 2006).

The presence of *HBZ* has also been suggested to promote T-lymphocyte proliferation (Arnold et al., 2008; Satou et al., 2006). Interestingly, the HBZ isoforms are able to modulate activator protein-1 (AP-1) activity and particularly interact with c-Jun (Basbous et al., 2003a; Hivin et al., 2007; Matsumoto et al., 2005). The formation of such a heterodimer prevents c-Jun from activating transcription of AP-1-dependent promoters and leads to a reduction in its DNA-binding activity (Basbous et al., 2003a; Hivin et al., 2006; Matsumoto et al., 2005). It has been proposed that this inhibition of the c-Jun DNA-binding activity by HBZ-SP1 could be responsible for the HBZ-SP1 repressor activity on c-Jun. However, we have recently demonstrated that HBZ-SP1 can mediate the sequestration of JunB, another Jun family member, to particular nuclear bodies (that we have termed HBZ-NBs) corresponding to transcriptionally-inactive sites (Hivin et al., 2007). In the light of this new data, it was important to determine if the negative effect of HBZ-SP1 on c-Jun activity was due to the weak DNA-binding activity of the HBZ-SP1/c-Jun heterodimer or to its sequestration to HBZ-NBs. To answer this question, we generated a mutant for which specific residues present in the modulatory (MD) and DNA-binding domain (DBD) of HBZ-SP1 were substituted for the corresponding c-Fos amino acids and its effect was tested on c-Jun activity. By this approach, we demonstrate that the repression of c-Jun activity *in vivo* is a consequence of the HBZ-SP1-mediated sequestration of c-Jun to the HBZ-NBs.

Results

Construct and analysis of the mutant HBZ-SP1-mutMD/DBD

HBZ-SP1 is a prototypical bZIP transcriptional factor (Fig. 1), with an N-terminal transcriptional activation domain, a central domain involved in its nuclear localization, and a C-terminal bZIP domain (Clerc et al., 2008; Gaudray et al., 2002; Hivin et al., 2005). However, HBZ-SP1 DBD diverges from the consensus basic subdomain bb-bN–AA-b(C/S)R-bb present in the bZIP domain of certain cellular transcriptional factors (Johnson, 1993; Kouzarides and Ziff, 1988; Vinson et al., 1989). In addition, we have recently demonstrated that HBZ-SP1 contains a cluster of amino acids, termed MD (Fig. 1), composed of basic and acidic residues immediately adjacent to DBD and involved in the modulation of Jun transcriptional potency (Hivin et al., 2006). We had then concluded that both HBZ-SP1 domains, MD and DBD, were responsible for the HBZ-SP1-mediated inhibition of c-Jun activity. If this conclusion is correct, we should be able to transform HBZ-SP1 into a transcriptional activator of c-Jun by mutating its MD and DBD regions. In order to test this idea, specific residues present in the HBZ-SP1 MD and DBD domains were substituted for the corresponding c-Fos amino acids. Indeed, unlike HBZ-SP1, c-Fos, a typical heterodimer partner of c-Jun, has been demonstrated to stimulate the *trans*-activating potential of c-Jun through its MD and DBD regions (Neuberg et al., 1991; Ryseck and Bravo, 1991). The structure of the new mutant, named here HBZ-SP1-mutMD/DBD, is shown in Fig. 1. Because N, A, and R have been described to be highly conserved (N–AA-b(C/S)R) in cellular bZIP factor DBD (Fig. 1), these residues were first reintroduced in the HBZ-SP1 basic motif (A–AK-HSA replaced with N–AA-KSR). However, we have previously demonstrated that these mutations are not sufficient (Hivin et al., 2006). For this reason, additional substitutions were generated in the HBZ-SP1 basic motif as already described (Hivin et al., 2006) to create an efficient DBD (as shown in Fig. 1). MD was also modified since c-Fos has been demonstrated to stimulate the *trans*-activating potential of c-Jun through not only its DBD but also its MD (Neuberg et al., 1991; Ryseck and Bravo, 1991).

First, *in vivo* expression of HBZ-SP1-mutMD/DBD was compared to the wild type HBZ-SP1 (HBZ-SP1-WT) by cloning their cDNA into a mammalian expression vector derived from pIRES2-EGFP (Clontech). This vector gives rise to proteins under study (here HBZ-SP1-WT and HBZ-SP1-mutMD/DBD) and enhanced-green-fluorescent protein (EGFP) from the same bicistronic mRNA. EGFP serves as an internal standard for normalization and allows the comparison of the level of expression for both HBZ-SP1 forms. Moreover, our vector permits Myc tagging of HBZ-SP1 proteins at the N-terminus. Protein levels were determined by immunoblotting and, as shown in Fig. 2A, the mutant was stably expressed. We then analyzed the ability of HBZ-SP1-mutMD/DBD to interact with c-Jun by a yeast two-hybrid assay. By this approach, we found that the mutations introduced in HBZ-SP1 MD and DBD sequences did not alter the interactions with c-Jun (Fig.

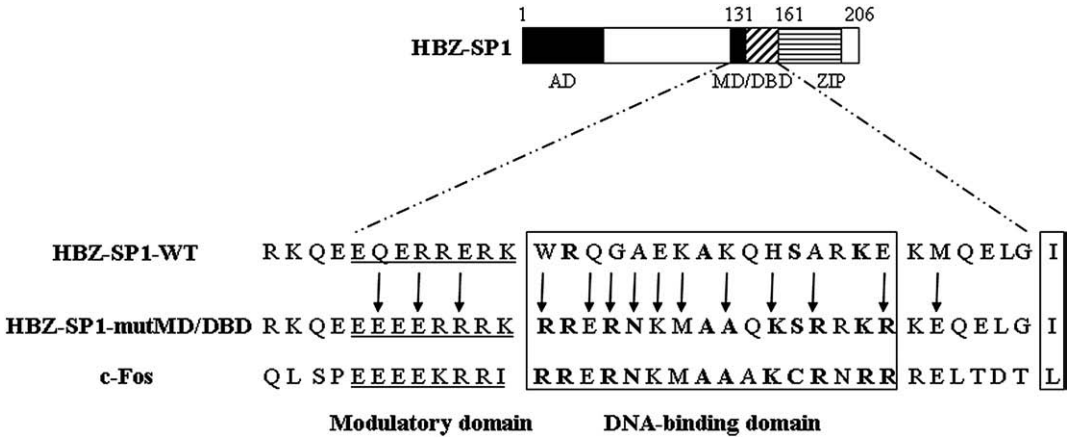

Fig. 1. Structure of HBZ-SP1-mutMD/DBD. HBZ-SP1 possesses an activation domain (AD), a modulatory domain (MD), and a bZIP structure, which includes a DNA-binding domain (DBD) and a leucine zipper (ZIP). The wild type HBZ-SP1 (HBZ-SP1-WT), the mutated HBZ-SP1 (HBZ-SP1-mutMD/DBD), and c-Fos modulatory and DNA-binding domains are aligned. MD sequences are underlined; DBD and the first ZIP residues are in boxes. The conserved residues of the consensus basic motif (bb-bN–AA-b(C/S)R-bb) are in bold. Arrows indicate HBZ-SP1-residues mutated to corresponding c-Fos amino acids.

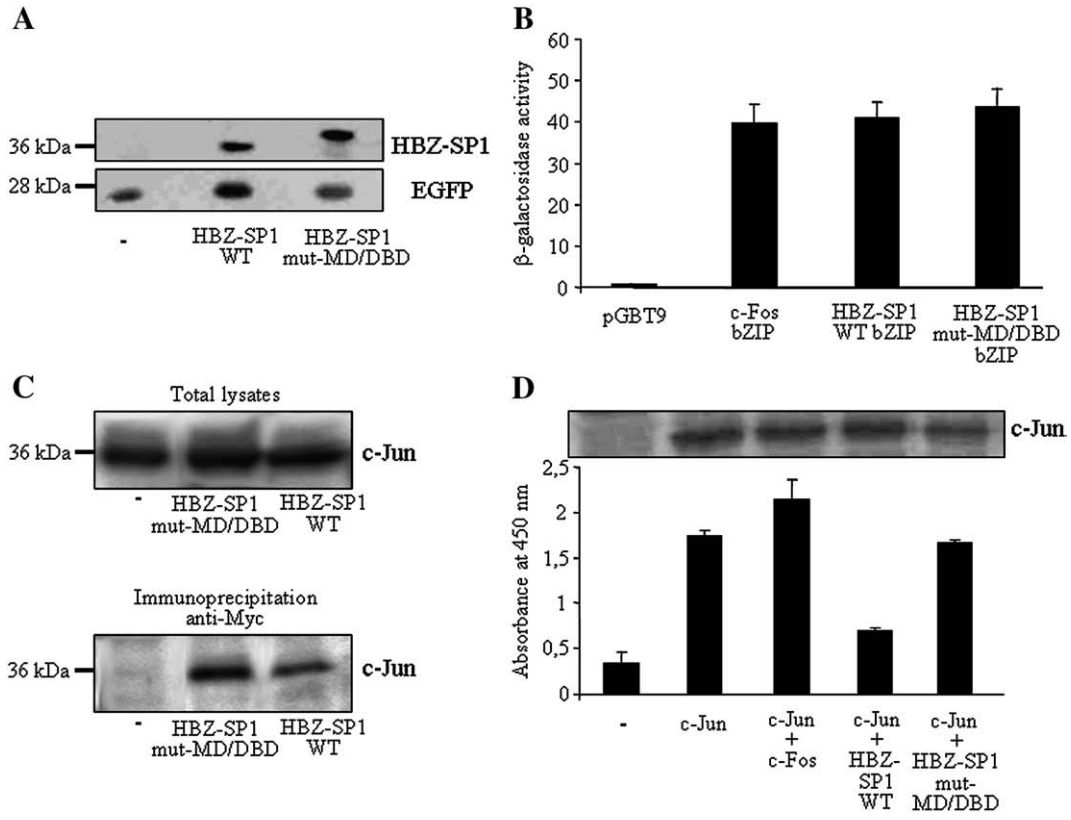

Fig. 2. DNA-binding and *trans*-activation potential of c-Jun/HBZ-SP1-mutMD/DBD complex. (A) Expression of HBZ-SP1-WT and HBZ-SP1-mutMD/DBD *in vivo*. Expression of the proteins in 293T cells was detected by western blotting using total cell extracts. HBZ-SP1 proteins and EGFP were visualized using the 9E10 anti-Myc monoclonal antibody and an anti-EGFP antiserum, respectively (- : cells transfected with the empty pIRES2-EGFP-Myc plasmid). Molecular size markers are indicated in kiloDaltons (kDa) on the left. (B) Interaction study between HBZ-SP1-mutMD/DBD and c-Jun by yeast two-hybrid assays using a liquid culture β-galactosidase assay. Yeasts were transformed with the expression vector pGAD containing the entire coding sequence of c-Jun fused to the GAL4 activation domain along with pGBT9 expressing the GAL4 DNA-binding domain fused to the region encompassing residues 120 to 206 from c-Fos, HBZ-SP1, or the mutant. The β-galactosidase activity was measured for three independent colonies per transformation assay using ONPG as a substrate. Mean values presented in the graph are expressed in Miller units. (C) HBZ-SP1-mutMD/DBD interacts with c-Jun *in vivo*. 293T cells were transfected with expression vectors pcDNA-c-Jun in the presence of empty pcDNA3.1(−)/Myc-His or pcDNA3.1(−)/Myc-His encoding Myc-tagged HBZ-SP1-mutMD/DBD or the wild type HBZ-SP1. (- : cells transfected with the empty pcDNA3.1(−)/Myc-His). Proteins from total lysates were directly probed with rabbit anti-c-Jun (Western blot shown above) or immunoprecipitated with mouse anti-Myc tag antibody, followed by Western analysis with rabbit anti-c-Jun (Western blot below). (D) DNA-binding activity of HBZ-SP1-mutMD/DBD. Microwells containing the AP-1-binding probe were incubated with nuclear extracts of 293T cells cotransfected with 4 μg of pcDNA-c-Jun and 4 μg of the vector expressing c-Fos, HBZ-SP1-WT, or HBZ-SP1-mutMD/DBD. Negative and positive controls corresponded to cells transfected with the pcDNA empty vector and pcDNA-c-Jun alone, respectively. The data represent the means of three values ± S.D. In the panel above, immunoblotting of nuclear proteins from transfected 293T cells was conducted using anti-c-Jun antibodies.

2B). However, the interaction between c-Jun and HBZ-SP1-mutMD/DBD was also studied by cotransfecting 293T cells with the expression vectors pcDNA-c-Jun and pcDNA3.1(−)/Myc-His encoding HBZ-SP1-WT or HBZ-SP1-mutMD/DBD. This vector permits Myc tagging of the viral proteins at the C-terminus. Cell extracts were then immunoprecipitated with anti-Myc tag monoclonal antibody, followed by Western analysis using rabbit anti-c-Jun antiserum. As shown in Fig. 2C, we found that c-Jun was immunoprecipitated with HBZ-SP1-mutMD/DBD and HBZ-SP1-WT confirming that the mutant is capable of interacting with c-Jun *in vivo*.

We also studied the DNA-binding activity of c-Jun in the presence of HBZ-SP1-mutMD/DBD. To evaluate DNA binding, we used the microwell colorimetric assay from Active Motif Europe (Renard et al., 2001), which is a highly sensitive non-radioactive DNA-binding ELISA. We had previously used this approach for the study of complex formation between c-Jun, HBZ-SP1, and the AP-1 motif (Basbous et al., 2003a; Hivin et al., 2006). Briefly, nuclear extracts of 293T cells transfected with c-Jun and either c-Fos, HBZ-SP1-WT, or HBZ-SP1-mutMD/DBD expression vectors were incubated in the presence of a double-stranded oligonucleotide containing the AP-1 site immobilized on a microwell plate. The DNA-binding activity was then measured by colorimetric assay using mouse anti-c-Jun antibodies. The ability of c-Jun to bind to the AP-1 motif was

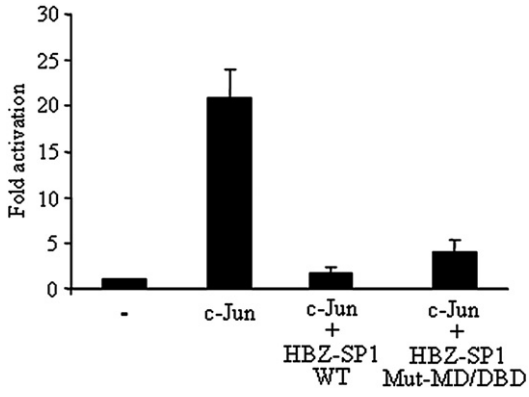

Fig. 3. HBZ-SP1-mutMD/DBD inhibits the *trans*-activation potential of c-Jun. CEM cells were cotransfected with 2 μg of a vector containing the luciferase reporter gene driven by the collagenase promoter, 5 μg of pcDNA3.1-*lacZ*, 1 μg of pcDNA-c-Jun, and 2 μg of pcDNA3.1(−)/Myc-His expressing HBZ-SP1-WT or HBZ-SP1-mutMD/DBD. Luciferase values are expressed as fold increases relative to values measured in cells transfected with pcDNA3.1(−)/Myc-His in the presence of the luciferase reporter vector. The total amount of DNA in each series of transfection was kept constant by the addition of the needed quantity of empty plasmids. Luciferase values were normalized for β-galactosidase activity. Values represent the mean ± S.D. ($n = 3$).

different since HBZ-SP1-WT decreased c-Jun binding while c-Jun showed a significant affinity for the AP-1 binding motif in the presence of HBZ-SP1-mutMD/DBD (Fig. 2D). This result was expected since amino acid substitutions were introduced in the sequence of the DBD basic subdomain of HBZ-SP1.

HBZ-SP1-mutMD/DBD still inhibits c-Jun transcriptional activity

We next examined the effect of HBZ-SP1-mutMD/DBD on transcription driven by the collagenase promoter that contains a canonical AP-1 element. The reporter plasmid was cotransfected in CEM cells with pcDNA-c-Jun in the presence of the mammalian expression vector pcDNA3.1(−)/Myc-His encoding HBZ-SP1-WT or HBZ-SP1-mutMD/DBD. As shown in Fig. 3, expression of c-Jun alone activated expression of the luciferase reporter gene by 21-fold, but this stimulation was inhibited not only in the presence of HBZ-SP1-WT but also of HBZ-SP1-mutMD/DBD. These data suggested that the modification of HBZ-SP1 MD and DBD sequences by the corresponding c-Fos domains was not sufficient to counteract the negative effect of HBZ-SP1 on c-Jun activity. However, as DBD has been shown to be involved in the nuclear transport of the viral protein (Hivin et al., 2005), the absence of a *trans*-activation potential by HBZ-SP1-mutMD/DBD could be an aberrant subcellular localization of this mutant. Its subcellular distribution was therefore studied by immunofluorescence microscopy using a mouse anti-Myc antibody in COS cells transfected with the Myc-tagged HBZ-SP1-mutMD/DBD expression vector pcDNA3.1(−)/Myc-His. As shown in Fig. 4A, HBZ-SP1-mutMD/DBD did localize to the nucleus. Interestingly, the mutant also accumulated in HBZ-NBs as we had previously described for HBZ-SP1-WT (Hivin et al., 2005, 2007).

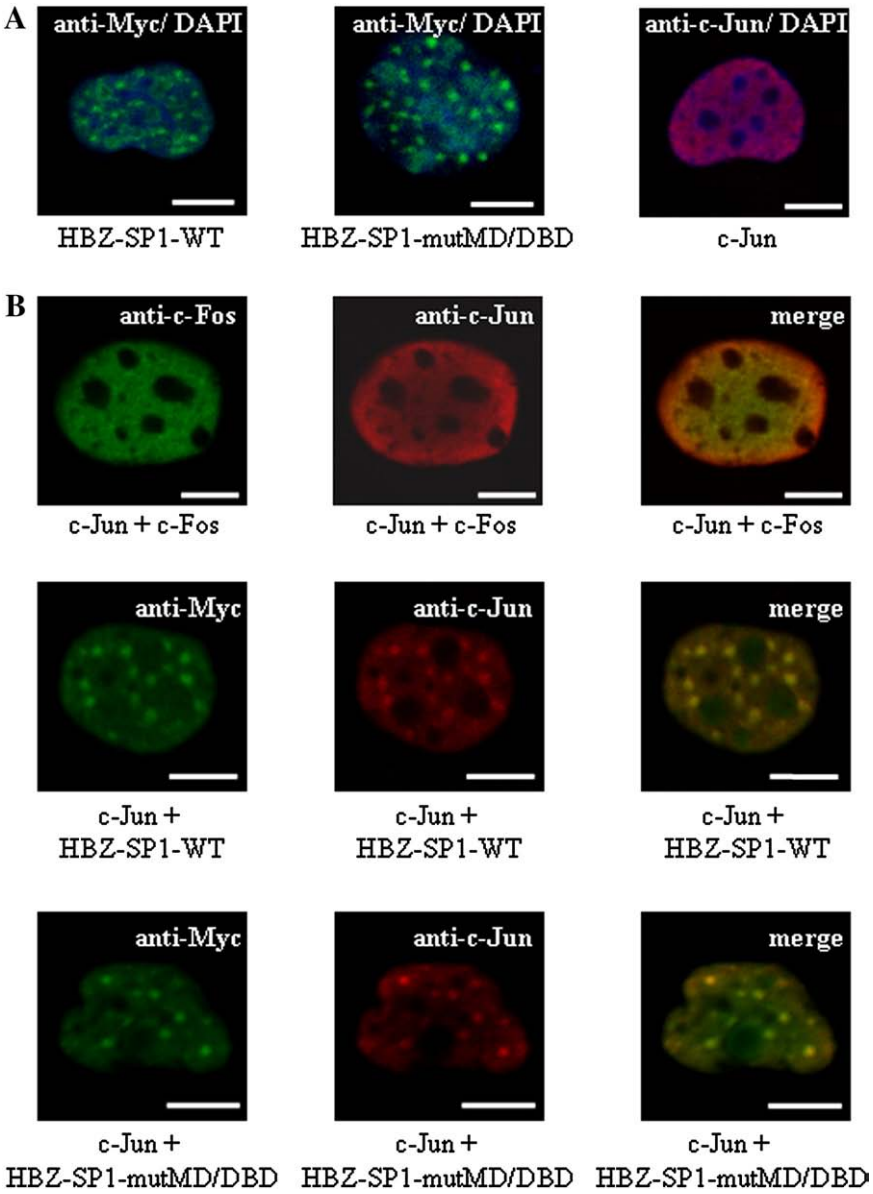

Fig. 4. Subnuclear localization of HBZ-SP1-mutMD/DBD in COS cells. (A) HBZ-SP1 mutMD/DBD accumulates in HBZ-NBs. Expression vectors for HBZ-SP1-WT, HBZ-SP1-mutMD/DBD, and c-Jun were transiently transfected into COS cells. Cells were cultivated on glass sides, fixed, and treated with Vectashield containing DAPI for direct characterization of the nucleus by confocal microscopy. The localization of the Myc-tagged chimeras was analyzed using the mouse anti-Myc antibody and goat anti-mouse IgG antibodies coupled to FITC. c-Jun was detected using a rabbit anti-c-Jun antibody and goat anti-rabbit IgG antibody coupled to Texas Red. (B) HBZ-SP1-mutMD/DBD colocalizes with c-Jun. COS cells were cotransfected with expression vectors for c-Jun in the presence of c-Fos, HBZ-SP1-WT, or HBZ-SP1-mutMD/DBD. The viral proteins and c-Jun were detected as described above. The localization of c-Fos was analyzed using mouse anti-c-Fos antibody and goat anti-mouse IgG antibodies coupled to FITC. Analyses of the green, red, and merged fluorescent signals were performed by confocal microscopy. The white bars correspond to a scale of 10 μm.

HBZ-SP1-mutMD/DBD colocalizes with c-Jun in HBZ-NBs

We have recently demonstrated that HBZ-SP1-mediated sequestration of JunB to the HBZ-NBs might be causing the repression of JunB activity *in vivo* (Hivin et al., 2007). Therefore, we conducted a comparison between the staining pattern of c-Jun induced by HBZ-SP1-WT and by HBZ-SP1-mutMD/DBD. In the presence of c-Fos, c-Jun showed a diffuse signal in the nuclei of transfected COS cells (Fig. 4B) while it was targeted to HBZ-NBs in the presence of HBZ-SP1-WT (Fig. 4B). Interestingly, a similar distribution was observed in the presence of HBZ-mutMD/DBD (Fig. 4B). Hence, our results strongly argue that HBZ-SP1 inhibits c-Jun activity by sequestration to HBZ-NBs as already described for JunB (Hivin et al., 2007). Moreover, they suggest that the mutations in the HBZ-SP1 MD and DBD sequence are not sufficient to transform HBZ-SP1 into an activator of c-Jun because HBZ-SP1-mutMD/DBD remains able to target c-Jun into HBZ-NBs.

We had previously constructed a HBZ-SP1/c-Fos chimera, termed H14F (Hivin et al., 2006) that has a structure identical to HBZ-SP1-mutMD/DBD except for its ZIP motif. Indeed, H14F possesses the ZIP motif of c-Fos while HBZ-SP1-mutMD/DBD contains that of HBZ-SP1 (Fig. 5A). Interestingly, as shown in Fig. 5B, H14F stimulates c-Jun transcriptional activity (Hivin et al., 2006). For this reason, the nuclear distribution of c-Jun was studied in the presence of H14F. When H14F and c-Jun were coexpressed in COS cells, H14F colocalized with c-Jun and both proteins were diffusely distributed throughout the nucleus (Fig. 5C). This observation supports the notion that the ZIP motif of HBZ-SP1 is involved in the repression of c-Jun activity by targeting the cellular factor into the HBZ-NBs.

It has been proposed that the HBZ isoform produced by the non-spliced mRNA inhibits c-Jun activity by promoting c-Jun degradation through a proteasome-dependent pathway (Matsumoto et al., 2005) while HBZ-SP1 has been found to have less ability to degrade c-Jun (Isono et al., 2008). Therefore we also studied the nuclear

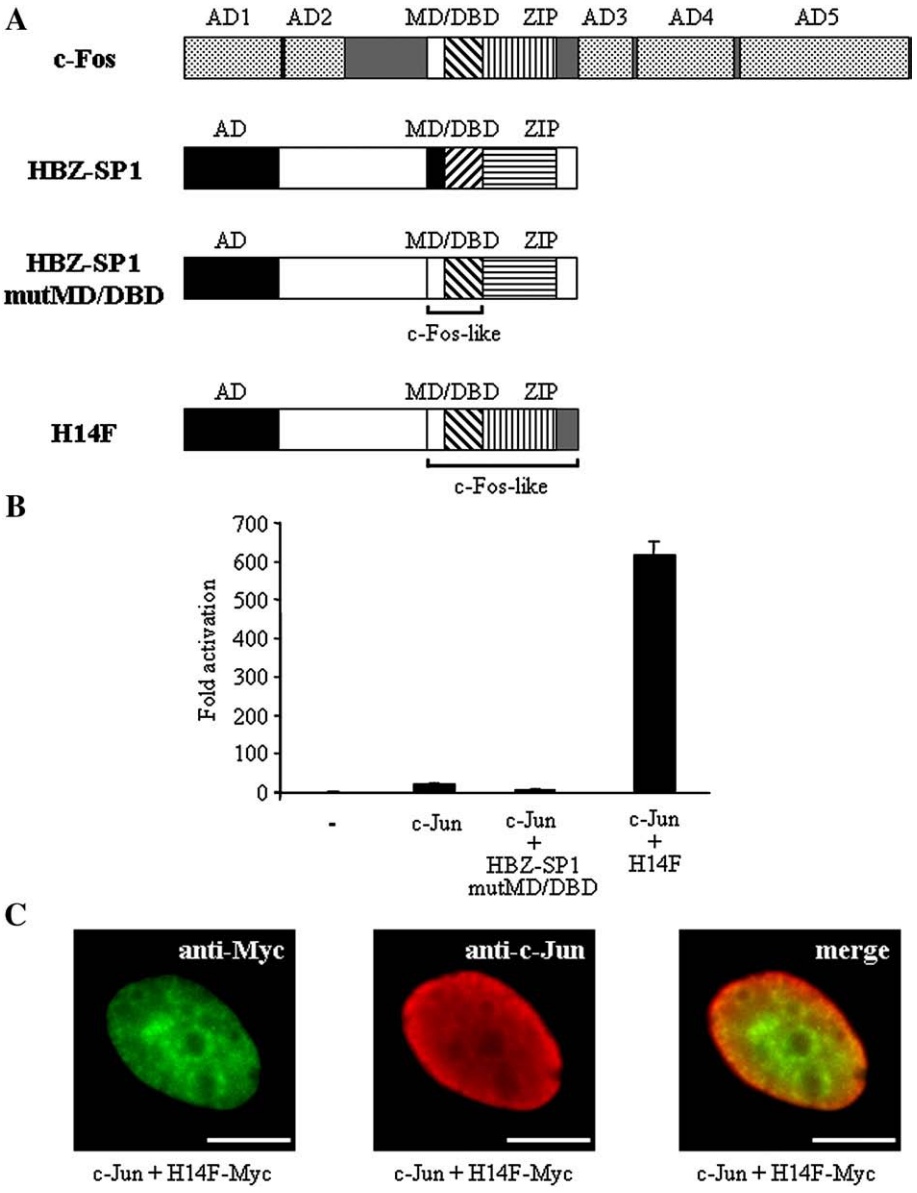

Fig. 5. H14F does not form HBZ-NBs in the presence of c-Jun. (A) Comparison of the structures of HBZ-SP1-mutMD/DBD and H14F and (B) their effect on the *trans*-activation by c-Jun. Expression vectors for HBZ-SP1-mutMD/DBD or H14F were cotransfected with a c-Jun expression vector together with a vector containing the luciferase reporter gene driven by the collagenase promoter as described in the legend of Fig. 3. (C) Immunofluorescence microscopy analysis of the colocalization of c-Jun and H14F *in vivo*. COS cells cotransfected with pcDNA-c-Jun and pcDNA-H14F-Myc were analyzed as described in the legend of Fig. 4. Analyses of the green, red, and merged fluorescent signals were performed by fluorescence microscopy. The white bars correspond to a scale of 10 μm.

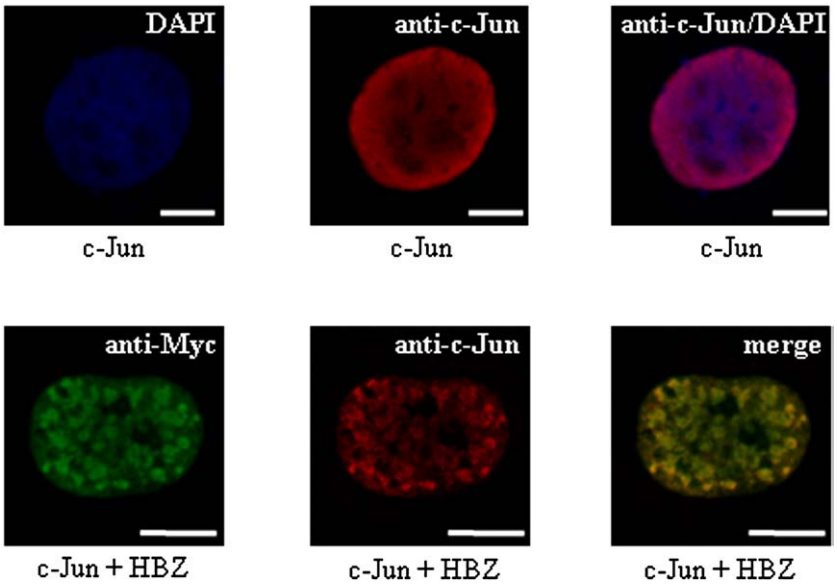

Fig. 6. Confocal microscopy analysis of the colocalization of c-Jun and the HBZ isoform. COS cells cotransfected with pcDNA-c-Jun and pcDNA-HBZ-Myc were labelled as described in the legend of the Fig. 4. Analysis of the green, red, and merged fluorescent signals was performed by confocal microscopy. The white bars correspond to a scale of 10 μm.

distribution of c-Jun in the presence the HBZ isoform (Fig. 6). The staining pattern of c-Jun was modified since the viral protein was able to entail a redistribution of c-Jun into HBZ-speckled structures already described by us and corresponding to transcriptionally-inactive sites (Hivin et al., 2005).

Discussion

The AP-1 transcription complex is involved in a multitude of cellular processes such as proliferation, differentiation, and cell death. In unstimulated T cells, the basal AP-1 level is low but there is a rapid induction of AP-1 activity after T-cell stimulation. The AP-1 transcription complex has been shown to be involved in the regulation of numerous cellular genes involved in lymphocyte activation, such as the *IL-2* gene (Foletta et al., 1998). Interestingly, high levels of AP-1 activity has been detected in HTLV-I-infected T-cell lines (Iwai et al., 2001; Mori et al., 2000), with increased levels of mRNAs encoding c-Jun, JunB, JunD, c-Fos, and Fra-1 (Fujii et al., 1991; Hooper et al., 1991). In addition, it has been suggested that Tax could be involved in AP-1 activation through the phosphatidylinositol 3-kinase/Akt pathway (Peloponese and Jeang, 2006). On the other hand, high constitutive activation of AP-1 has also been described in primary non Tax-expressing primary ATL cells, in which active AP-1 complexes contain JunD but not c-Jun and JunB (Mori et al., 2000), suggesting that c-Jun and JunB are not active in ATL cells.

Interestingly, non-spliced and spliced HBZ mRNAs (involved in the production of the HBZ and HBZ-SP1 isoforms, respectively) have been reported to be detectable in almost all tested ATL cells (Murata et al., 2006; Satou et al., 2006; Usui et al., 2008). Both isoforms are able to modulate AP-1 activation by binding to the different members of the Jun family (Basbous et al., 2003a; Hivin et al., 2006, 2007; Matsumoto et al., 2005). Furthermore, their interaction with c-Jun and JunB leads to a reduction in their transcriptional activity (Basbous et al., 2003a; Hivin et al., 2006, 2007; Matsumoto et al., 2005). We have previously demonstrated that HBZ-SP1 inhibits JunB transcriptional activity by targeting JunB to HBZ-NBs, which correspond to inactive sites of transcription (Hivin et al., 2005, 2007). Herein, we confirm that HBZ-SP1-mediated sequestration of c-Jun to the HBZ-NBs may also be causing the repression of c-Jun activity. Indeed, we generated a mutant, HBZ-SP1-mutMD/DBD, for which specific residues present in the MD and DBD of HBZ-SP1 were substituted for corresponding amino acids of c-Fos. Although this mutant in the presence of c-Jun showed a significant *in vitro* affinity for the AP-1 binding site, it remained unable to stimulate transcription of the AP-1-dependent promoter of the collagenase gene *in vivo*. On the other hand, when the bZIP domain of this mutant was replaced by the corresponding ZIP domain of c-Fos (HBZ-SP1-mutMD/DBD modified into H14F), the heterodimer H14F/c-Jun bound to the AP-1 binding sequence *in vitro* (Hivin et al., 2006) and was further capable of highly activating AP-1-dependent promoter transcription *in vivo*. We have previously observed that the ZIP domain of HBZ-SP1 is involved in its nuclear trafficking and that its association with JunB leads to the formation of HBZ-NBs (Hivin et al., 2007). We show here that c-Jun also forms HBZ-NBs in the presence of HBZ-SP1-mutMD/DBD while this transcription factor is diffusely distributed throughout the nucleus in the presence of H14F, which is identical to HBZ-SP1-mutMD/DBD except for its ZIP domain. Moreover, we have already demonstrated that in the presence of JunD, HBZ-SP1 is also diffusely distributed throughout the nucleoplasm and that no HBZ-NBs are formed in this situation (Hivin et al., 2007). Interestingly, JunD is the only member of the Jun family that can be activated by HBZ-SP1 (Kuhlmann et al., 2007). Taken together, our results suggest that HBZ-SP1 inhibits c-Jun and JunB *trans*-activating capacity *in vivo* mainly by their sequestration to the HBZ-NBs.

The HBZ isoform produced by the non-spliced mRNA is also capable of inhibiting c-Jun by promoting its proteasomal degradation (Matsumoto et al., 2005). It has been suggested that HBZ would act as a tethering factor between the 26S proteasome and c-Jun (Isono et al., 2008). On the other hand, HBZ-SP1 has less ability to promote the degradation of c-Jun because its interaction with the proteasome is less efficient than that of HBZ (Isono et al., 2008). This difference is not unexpected since the N-terminal region of HBZ is involved in the regulation of c-Jun degradation (Matsumoto et al., 2005). It remains unclear why both isoforms would inhibit c-Jun by two different mechanisms. One possible explanation might be that the HBZ-NBs correspond to transient storage sites to control temporary c-Jun activity. HBZ-NBs containing HBZ would promote c-Jun degradation while HBZ-SP1-containing NBs would rather serve as passive storage sites for c-Jun. In the latter case, it is conceivable

that the stored c-Jun could be eventually recruited to active transcription loci according to the state of the infected T-cell. For the moment, this model remains hypothetical and still needs further experiments to be demonstrated.

Materials and methods

Plasmid constructs

The vectors pcDNA-HBZ-Myc, pcDNA-HBZ-SP1-Myc, pcDNA-H14F-Myc, pcDNA-c-Jun, and pcDNA-c-Fos constructs have previously been described (Cavanagh et al., 2006; Hivin et al., 2006). To generate HBZ-SP1-mutMD/DBD, DNA was amplified by PCR from the previously published H14F chimera (Hivin et al., 2006) and subcloned into pcDNA3.1(−)/Myc-His generating pcDNA-HBZ-SP1-mutMD/DBD-ΔZIP. The HBZ-SP1 ZIP DNA was then amplified from pcDNA-HBZ-SP1-Myc, digested with HindIII, and subcloned in frame into HindIII-linearized pcDNA-HBZ-SP1mutMD/DBD-ΔZIP to produce pcDNA-HBZ-SP1-mutMD/DBD. The artificially introduced HindIII site was then deleted to regenerate the proper HBZ-SP1 amino acid sequence. Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) and the construct was sequenced to ensure that no unintended mutations were introduced during PCR amplification. The pIRES2-EGFP-Myc plasmid was obtained from Jihane Basbous. The vector allows detection of the recombinant protein with an anti-Myc antibody. For the different constructs, amplified DNA was cloned into EcoRI/BamHI-digested pIRES2-EGFP-Myc. For yeast two-hybrid assays in yeast, amplified DNA was digested by EcoRI and cloned into pGBT9.

Analysis of protein expression by western blotting

293T cells were cultured in DMEM supplemented with 10% FCS and transfected with 7 μg of expression vector using the jetPEI™ transfection reagent (Qbiogene) according to the manufacturer's instructions. Protein extracts (100 μg) from transfected 293T cells were electrophoresed onto sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and blotted on polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were then incubated 1 h at room temperature in a blocking solution (phosphate-buffered saline [PBS] containing 5% milk) prior to addition of antiserum. After 1 h, membranes were washed three times with PBS-0.5% Tween 20 and further incubated with a peroxydase-conjugated goat anti-mouse IgG antibody for 1 h. After three washes, membranes were incubated with the enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech). Membranes were then exposed to hyperfilms-ECL (Amersham Pharmacia Biotech). Proteins with two Myc epitopes, tagged at their N-terminal end, were detected with the mouse anti-Myc antibody 9E10 (Sigma) while EGFP was revealed with a mouse anti-EGFP antibody (Clontech). Mouse anti-EGFP and anti-c-Jun antibodies were purchased from Clontech and Oncogene Research Products, respectively.

Immunoprecipitation assays

Immunoprecipitations were performed as already described (Clerc et al., 2008). Briefly, 293T were transfected with 5 μg of expression vector using the jetPEI™ transfection reagent (Qbiogene). Lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 8.0, 1% Triton X-100, 100 mM NaCl, 1 mM $MgCl_2$, 2 mM benzamidine, 2 μg/ml of leupeptin, 2 μg/ml of aprotinin, and 1 mM PMSF). Antibody-bound beads were washed in RIPA buffer and 200 μg of cell lysates were added to each antibody-bead suspension, incubated overnight, and washed several times in RIPA buffer. Bound proteins were analyzed on SDS-polyacrylamide gel and detected by Western blotting as described.

Microwell colorimetric AP-1 assays

Nuclear extracts (15 μg) of cotransfected 293T cells were incubated with 30 μl of binding buffer (10 mM HEPES pH 7.5, 8 mM NaCl, 12% glycerol, 0.2 mM EDTA, 0.1% BSA) in microwells coated with probes containing the AP-1-binding site (Trans-AM™ AP-1 of Active Motif Europe, Belgium). After 1 h incubation at room temperature, microwells were washed three times with PBS containing 0.1% Tween 20. The AP-1-bound complexes were detected with a mouse anti-c-Jun antibody followed by the addition of a peroxydase-conjugated antibody. For colorimetric detection, tetramethylbenzidine was incubated at room temperature before addition of the stop solution. Optical density was read at 450 nm, using a 620 nm reference wavelength with a Tecan microplate reader.

Yeast two-hybrid assays

Interactions between HBZ-SP1-mutMD/DBD and c-Jun were analyzed by two-hybrid assay in the *Saccharomyces cerevisiae* strain HF7c (Wurch et al., 1990). Strain HF7c possesses the *Escherichia coli lacZ* gene driven by three copies of the *GAL4* consensus sequence. The region of the mutant containing both MD and bZIP domain (from amino acid 120 to 206) was cloned in frame with the GAL4 DNA-binding domain in the pGBT9 vector. Yeasts were cotransformed with pGBT9 and pGAD-c-Jun as previously described (Basbous et al., 2003b). The β-galactosidase assay was carried out in the presence of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate on three independent colonies per transformation according to the manufacturer's protocol (Clontech). The β-galactosidase activity was calculated in Miller units (Miller, 1972).

Cotransfections and luciferase assays

CEM cells were transiently cotransfected according to the previously published protocol (Gachon et al., 2001). The β-galactosidase-containing pcDNA3.1-*lacZ* vector (5 μg) was added in each transfection to control for transfection efficiency. The total amount of DNA in each transfection was kept constant through the addition of appropriate quantities of empty plasmids. Equal amounts of proteins from each cell extracts were then used for luciferase and β-galactosidase assays. Luciferase assays were performed in an automated luminometer with the Genofax A kit (Yelen, Ensue la Redonne, France) (Arpin-Andre and Mesnard, 2007).

Immunofluorescence microscopy analysis

COS cells were cultured in DMEM supplemented with 10% FCS. Cells were then seeded onto glass slides and, after 24 h, transfected using the jetPEI™ transfection reagent (Qbiogene). At 48 h post-transfection, cells were washed with PBS, fixed, and permeabilized with 4% paraformaldehyde and 0.1% Triton X-100 for 10 min at room temperature. Cells were incubated with primary antibody (mouse anti-Myc antibody 9E10, Sigma, or rabbit anti-c-Jun, Santa Cruz Biotechnology Inc.) for 1 h at room temperature. The same approach was carried out with COS cells transfected with pcDNA-c-Fos labelled with mouse anti-c-Fos antibody from Santa Cruz Biotechnology.

Samples were subsequently washed with PBS and then incubated with secondary FITC- or Texas Red-labelled antibodies (Pierce) for 1 h at room temperature. Coverslips were mounted with the Vectashield reagent containing DAPI (Abcys) for direct observation. Fluorescence images were acquired by fluorescence microscopy (model DM R; Leica) at room temperature with a 63×, NA 1.32, oil immersion objective at pinhole size 1 Airy (observation with immersion oil type

DF, Cargille Laboratories Inc.). DAPI, FITC, and Texas Red were excited by 365-, 492-, and 596-nm laser light and emission was detected at 420, 520, and 620 nm, respectively. Analysis of the green, red, and yellow fluorescence for colocalization experiments was performed with a Bio-Rad MRC 1024 confocal microscope.

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